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Co-localization of *N*-methyl-D-aspartate receptors and substance P (neurokinin-1) receptors in rat spinal cord

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Abstract

Glutamate, substance P (SP), and their receptors have been implicated in the initiation and maintenance of persistent pain through an interaction at second order spinal cord neurons. Employing well-characterized antibodies to the SP receptor and the *N*-methyl-D-aspartate receptor (NR1 subunit, splice variant missing exon 22), we demonstrate co-localization of these receptors on second order neurons at cervical, thoracic, lumbar, and sacral spinal cord levels. The co-localization was marked in lamina I of the dorsal horn at all levels and in the intermediolateral nucleus of the thoraco-lumbar spinal cord nuclei associated with autonomic function.

Keywords: Glutamate, Pain; Onuf's nucleus; Motorneurons; Intermediolateral nucleus; Sympathetic nervous system; Parasympathetic nervous system; Receptor co-localization

There are at least two major groups of neuroactive substances co-localized in nociceptive primary afferent terminals: neuropeptides, (e.g. substance P (SP), substance K, α- and β-calcitonin gene-related peptides) and excitatory amino acids (glutamate, aspartate). These transmitters interact with receptors on second order neurons in spinal cord and the present study examines their cellular co-localization by double-staining immunocytochemistry.

Excitatory amino acid receptors have been broadly categorized into ionotropic and metabotropic groups. The eight metabotropic receptor subtypes transduce information via second messengers. The ionotropic group includes kainate, (±)-αamino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA), and *N*-methyl-D-asparate NMDA receptors [1,10,18]. Pharmacological studies show the involvement of NMDA receptors in hyperalgesia, 'wind-up', and central sensitization, all of which contribute to the initiation and maintenance of persistent and neuropathic pain [8,12,17]. Treatment with NMDA antagonists produces an anti-hyperalgesic effect without affecting baseline nociception, suggesting NMDA receptors selectively enhance spinal excitability in persistent pain states [3].

The NMDA receptor contains both NR1 and NR2 subunits. The NR1 gene contains 22 exons and undergoes alternative splicing of exons 5, 21, and 22 to yield seven different variants [18]. When exon 22 is spliced out, the 3′ untranslated region provides a short open reading frame that yields a new 22 amino acid C-terminus (NR1 22-). The NR1 splice variants are conserved in mouse, rat and humans and the presence or absence of the three exons profoundly affects receptor and channel conductance activity [5,18].

SP or neurokinin 1 receptors are widespread in the central nervous system (CNS) [2] and SP participates in motor, autonomic and sensory functions. The present report will refer to the NK1 receptor as substance P receptor (SPR) to distinguish it from the NR1 subunit. SP and SPR are particularly abundant in lamina I of the spinal cord [2,16]. In persistent pain states, interactions are thought to occur between SPR and NMDA receptors. SPR immunocytochemical staining yields a reaction product localized to the plasma membrane and causes the soma and dendritic processes to be outlined by reaction product [16]. In contrast, a large fraction of the NR1 22- variant is located in an intracellular pool. The different subcellular localizations allow the question of co-localization to be examined.

Five male Sprague–Dawley rats (Harlan, Indianapolis,

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IN) weighing 300 g were used. A unilateral inflammation was produced by subcutaneous injection of 6 mg carrageenan in 150 μl of sterile saline into the plantar aspect of the left hind limb. At 2 days post-injection, rats were transcardially perfused under deep pentobarbital anesthesia with ice cold saline and then ice cold 4% paraformaldehyde in phosphate buffered saline (PBS). Experimental protocols were approved by the NIDCR Animal Care and Use Committee.

The SPR antiserum has been described [16]. The immunogen for the NR1 22- splice variant used the unique peptide sequence, *N*-acetyl-QYHPTDITGPLNLSDPSCamide, found in the C-terminus [14] conjugated to keyhole limpet hemocyanin (Calbiochem, La Jolla, CA) via the C-terminal cysteine. An antiserum was raised in a rabbit and antigen affinity purified. This antiserum yields: (a) one major band of immunoreactivity at ~117 kDa on western blot corresponding to the M_r of the NR1 subunit, (b) one major band using extracts of chinese hamster ovary cells transiently transfected with a plasmid expressing the NR1 22- splice variant, and (c) a prominent immunocytochemical reaction product in the neuronal perikarya. The staining with the exon 22- antibody was completely blocked by preincubation and inclusion of 10^{-6} M target peptide in the primary antibody labeling steps; staining was not blocked with the same amount of exon 22+ peptide *N*-acetyl PRRAI EREEGQLQLCamide.

Tissues were post-fixed overnight, and cryoprotected in 30% sucrose. Thirty micron tissue sections were cut with a cryostat, blocked for 60 min in 3% normal goat serum (NGS), 0.75% Triton X-100 and incubated in the SPR antiserum (1:250 000 in 1% NGS/0.3% Triton) for 48 h. Sections were washed eight times in PBS over 15 min (8× washed) then incubated with biotinylated goat-antirabbit secondary antiserum (1:4000) for 45 min. The sections were incubated with avidin-biotinylated horseradish peroxidase (HRP) complex (Vector Labs Inc., Burlingame, CA), 8× washed and visualized using tyramide signal amplification (TSA) with reagents from NEN Life Sciences Products (Boston, MA). When employing TSA, sections that had reached the HRP step were incubated for 10 min in biotinylated tyramide (1:200) in 0.005% H_2O_2. The reaction catalyzed by HRP covalently attaches biotinylated tyramide to proteins in the vicinity of the peroxidase enzyme. Following 8× washes the sections were re-incubated with avidinbiotinylated HRP complex and visualized with nickel intensified 3,3′ diaminobenzidine tetrahydrochloride (DAB, 0.05%), containing 0.01% H_2O_2 in 0.1 M phosphate buffer. Sections were then incubated in NMDA NR1 22- receptor antiserum (1:2000 in 1% NGS/0.3% Triton) overnight, 8× washed, and incubated with secondary antibody as above. Following this, sections were 8× washed and incubated in avidin-biotinylated HRP complex for 30 min, and developed with DAB only. The above protocol showed no detectable cross-labeling between the cellular compartments. Other protocols examined, such as incubating with the NMDA NR1 antiserum first and employing TSA, or fluorescent techniques (with and without TSA) yielded an unacceptable degree of ‘cross-talk’.

SPR immunoreactivity (Ir) was restricted to the plasma membrane (Fig. 1A,C), while the reaction product for the NR1 22- antibody prominently labeled the perikarya and

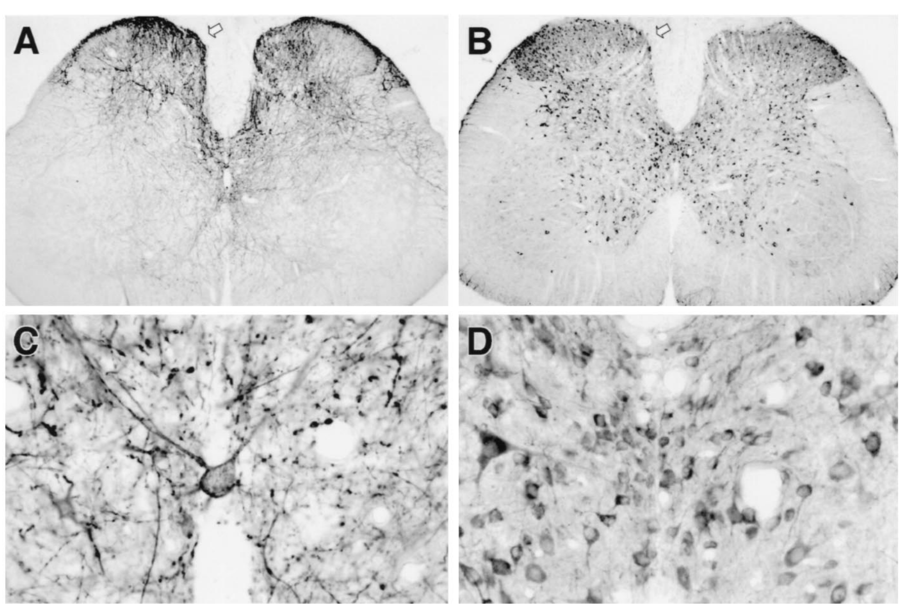

Fig. 1. (A) Lumbar spinal cord section from a rat with left hind paw inflammation, stained for SPR only. Note the increase in SPR-Ir in the left medial dorsal horn receiving input from the inflamed hind paw (open arrow) [9]. (B) Lumbar spinal cord section from a rat with left hind paw inflammation stained for NMDA NR1 22- subunit only. The open arrow denotes the left (inflamed) side. (C) Lamina X from section shown in (A), demonstrates localization of SPR to the plasma membrane of soma and dendrites. Note the large cell at the top of the central canal. (D) Lamina X from section shown in (B). Staining for NMDA NR1 22-subunit yields reaction product in the perikarya and proximal dendrites.

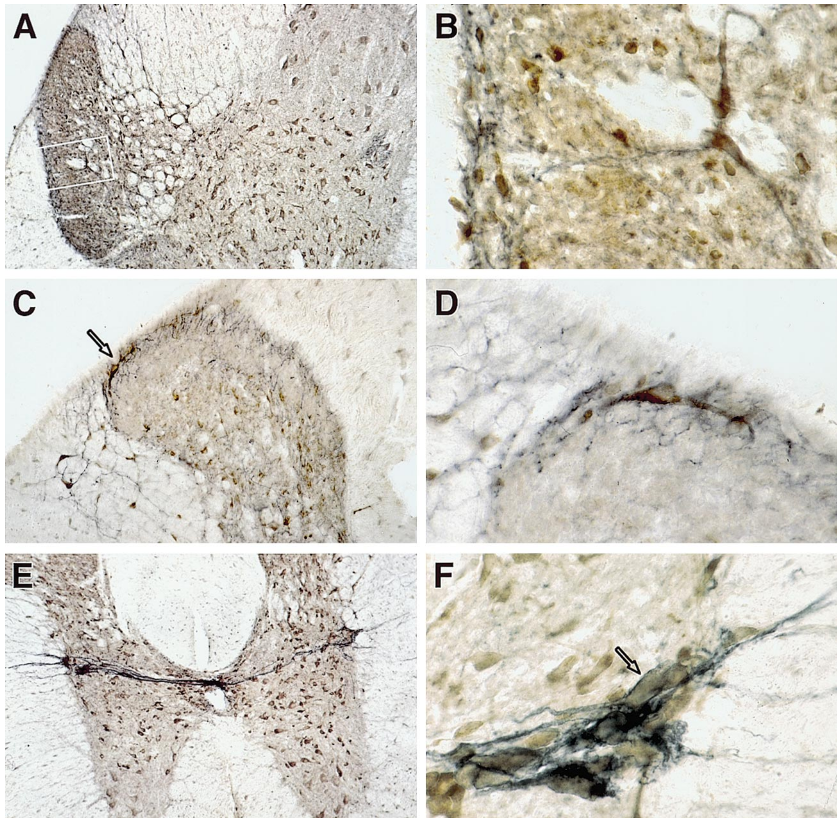

Fig. 2. Doubly stained sections (SPR and NMDA NR1 22- antibodies) from rat spinal cord. (A,B) Cervical level. Neurons in the lateral, reticulated zone of lamina V show receptor co-localization. In lamina III, a large doubly labeled neuron has dendritic processes that extend up to lamina I (boxed in white, see high power in panel B (630×)). (C,D) Thoracic level, dorsal horn. Cells in the lateral spinal nucleus and in lamina I (arrowed) show co-immunoreactivity. At high power (630×) the cell in lamina I resembles a Waldyer neuron and clearly co-localizes both receptor types (D). (E,F) Thoracic level. Intense SPR-NR1 co-localization is seen in neurons of the intermedio-lateral (IML) cell column, with SPR-Ir processes running laterally and medially across lamina X. High power (630×) of the IML (different section) shows cells with receptor co-immunoreactivity. SPR-Ir (black) is on the plasma membrane and the NMDA Ir (brown) is within the perikarya (see arrowed cell).

proximal dendritic shafts (Fig. 1B,D). The pattern of SPR-Ir observed duplicates previous studies [2,16]. Cells immunoreactive for NR1 22- were distributed throughout all the spinal laminae. A strong cluster of cells is seen dorsal to and on either side of the central canal (Fig. 1D). Positive cells are seen in the superficial layers of the dorsal horn. In the ventral horn the large motor neurons were non-immunoreactive in comparison to nearby smaller cells. The left side of the spinal cord in Fig. 1A,B receives input from the inflamed hind limb. An elevation in SPR-Ir can be seen with peripheral inflammation, consistent with previous SPR measurements using ribonuclease protection assays [9]. While the effect of inflammation is not the main subject of this report, an increase in NMDA NR1 22- Ir cells is also apparent in medial lamina I. The effect has been verified in a separate experiment and we are in the process of quantification of the increase.

The protocol employed resulted in no detectable cross-labeling using the antisera in the sequence described. We attribute this to nickel-intensified DAB visualization of the first immunogen: any cross-reaction at the second primary antibody stage deposits DAB onto nickel DAB, and the brown color does not show through the black. Indeed, we found many singly labeled cells for the NR1 subunit, (e.g. lateral to the central canal, singly labeled in Fig. 1B), and cells singly labeled for the SPR such as Onuf's nucleus in sacral cord (not shown, see Ref. [2]).

At all levels of the spinal cord SPR/NMDA receptors were co-localized in lamina I. Both small and large cells with strong co-immunoreactivity were detected (Fig. 2C). The large superficially located cells resembled Waldeyer neurons (Fig. 2D). Dendritic processes with SPR-Ir were seen in lamina I that arose from NMDA NR1 22- positive cells in lamina III (Fig. 2B). We consistently observed receptor co-localization in laminae IV and V, especially in neurons in the lateral, reticulated area of lamina V (Fig. 2A). Cells in the region of the lateral spinal nucleus also show clear receptor co-localization (Fig. 2C). In addition, spinal level-specific staining patterns and areas of co-localization included the following. Cervical: a group of cells, located in the ventral horn exhibited SPR-Ir and NMDA NR1 Ir (Fig. 2A). We were unable, however, to detect co-Ir on the same cells. These cells are probably lamina IX motorneurons commonly found in cervical sections. Thoraco-lumbar: neurons of the intermediolateral (IML) cell column exhibited intense surface SPR-Ir, which co-localized with NR1 Ir in their perikarya (Fig. 2E,F). Sacral: in Onuf's nucleus we detected SPR-Ir as reported [2] but there was very little NR1 22- Ir (not shown).

The antiserum raised against the NMDA NR1 22- splice variant lacks exon 22 but may also lack exon 21, (i.e. 21-, 22-) [14,18]. In situ hybridization studies with splice variant-specific probes show that the NR1 subunit lacking both exons 21 and 22 is the more abundant isoform expressed in spinal cord [15]. The distribution of the NMDA NR1 22- receptor protein in the present study overlaps and more clearly defines results obtained with in situ hybridization [15]. The antiserum employed in this study is thus suitable for examining NMDA receptor co-localization, although more co-localization may have been obtained with an NMDA antibody to a region common to all NR1 subunits.

Persistent pain states following tissue or nerve injury induce a functional plasticity that has been linked to glutamate receptors, in particular the ionotropic group. In persistent pain states, NMDA receptors are thought to interact with SPR to produce secondary hyperalgesia and allodynia. The SPR is involved in the initiation, but not the maintenance, of mechanical allodynia in the rat suggesting a complex interaction between SPR and NMDA receptors [3,4,6,8,13]. Potentiation of neuronal NMDA responses by SP is thought to play an important role in nociception and persistent pain states [11]. Our data reinforce the idea that co-release of glutamate and SP at C-type primary afferent synapses activates NMDA and SP receptors. Furthermore, it has been suggested that NMDA receptor activation releases SP from nociceptive primary afferent endings [7], creating a potential positive feedback mechanism. These mechanisms predict the co-localization of glutamate and SP receptors on the same nociceptive neurons, which we demonstrate in the present study.

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